THE INTRACELLULAR DISTRIBUTION OF CALCIUM IN THE MUCOSA OF THE AVIAN SHELL GLAND

WAYNE HOHMAN and HARALD SCHRAER

From the Department of Biophysics, The Pennsylvania State University, University Park

ABSTRACT

The intracellular distribution of calcium has been studied in the mucosa of the avian shell gland, a tissue which transports large quantities of calcium during discrete time intervals. Ca^{45} was administered to hens either in a single dose followed by sacrifice 5 min later or in repeated doses over an extended period followed by sacrifice 2 hr or 24 hr after the last injection. Subcellular fractions were isolated by differential centrifugation and analyzed for Ca^{45}. The Ca^{45} was located principally in the particulate fractions; the concentration (CPM Ca^{45}/mg N) was highest in the mitochondrial fraction. Comparisons of (1) the Ca^{45} distribution in shell gland cells with that of liver cells, (2) the alterations which occur due to the phase of the egg laying cycle, (3) the effects due to the time elapsed since the last injection of Ca^{45}, and (4) the Ca^{45} distribution of the short term experiments with that of the long term experiments revealed that the mitochondrial fraction of the shell gland appeared to be active in the movement of calcium. The microsomal fraction showed increased values in CPM Ca^{45}/mg N when calcification was occurring, which may indicate that the subcellular components of this fraction have a role in calcium transport. The nuclear and supernatant fractions did not seem to be involved in the transport process. The implications of these results concerning the manner by which calcium may be controlled on a cellular level in this system are discussed.

INTRODUCTION

A study of metal distribution in the avian shell gland (uterus) demonstrated that the calcium content was correlated with the location of the egg in the oviduct (Schraer and Schraer, 1965). This observation led us to examine the intracellular distribution of calcium (Ca^{45}) in shell gland mucosal cells at the subcellular level. The distribution of calcium, as well as other metals, has been studied in isolated subcellular fractions of other tissues, such as rat liver (Griswold and Pace, 1956; Thiers and Vallee, 1957), chick breast muscle (Cosmos, 1964), and canine intestinal mucosa (Cassidy et al., 1965). The particulate fractions, in general, and notably the mitochondrial fraction, have been implicated as sites of calcium accumulation.

Since the shell gland of the domestic fowl transports calcium to the lumen of the oviduct at an average rate of about 1.7 mg/min during a 15 to 20 hr period (Hertelendy and Taylor, 1961), this tissue appeared to be ideally suited for such a study. By comparing the Ca^{45} distribution of shell gland cells with that of liver cells, which are not in themselves known to transport large quantities of calcium, and by observing this distribution during different phases of the laying cycle, we have provided additional information concerning the mode of calcium transport across cells.

METHODS

Regularly laying single-comb White Leghorn hens in their first year of laying were maintained on a standard laying ration, unlimited amount of water, and a regular artificial light schedule. Hens with eggs in different parts of the oviduct were selected for each experiment.

CA^{45} ADMINISTRATION: An intravenous injection of approximately 250 μc of Ca^{45} in 1 cc of distilled water was administered via the brachial vein with a heparinized disposable syringe. Completion of the injection marked time zero. After a 5 min exposure to Ca^{45} nine hens were sacrificed and were designated as short term experiments. Two long term experiments in which hens were given daily intramuscular injections of 100 μc of Ca^{45}, each for approximately 1 month, were also undertaken. One of the latter hens (Experiment A) received 28 injections, the last being approximately 2 hr before sacrifice. Another hen (Experiment B) received 30 injections, the last being approximately 24 hr before sacrifice. All hens were treated identically for the remainder of the procedure.

Preparation and Analysis of the Shell Gland

GENERAL PROCEDURE: The experimental animals were sacrificed by decapitation and bled maximally. The shell glands were quickly removed, placed in beakers immersed in an ice bath, and immediately transferred to a cold room (0 to 4°C) where all subsequent operations were performed until fractionation was complete. Each shell gland was cut open longitudinally from the isthmouterine junction to the vagina to expose the mucosa. Ca^{45}, shown from preliminary experiments to be present on the mucosal surface, was removed by blotting with filter paper. To obtain those cells most probably involved in calcium transport, the mucosal layer was scraped free from the muscle layers with a plate glass knife. This resulted in the use of about 11 to 16% of the whole shell gland tissue for fractionation and analysis. These scrapings, referred to as the mucosal cells, were weighed and suspended in 0.44 M (w/v) sucrose (Witter et al., 1955) to make a 1:10 (w/v) preparation, and homogenized in a glass tube that was immersed in ice water for 3 to 4 min at 940 rpm with a Potter-Elvehjem (1936) homogenizer fitted with a radially serrated Teflon pestle. One ml of this homogenate contained the subcellular material from 0.1 g of the original cells. Five ml of this material was removed for analysis as representative of the total unfractionated homogenate. The remainder of the homogenate was fractionated by differential centrifugation.

DIFFERENTIAL CENTRIFUGATION: The light-colored nuclear fraction was sedimented at 755 g[1] for 15 min. This pellet was washed twice by resuspending it in one half the original volume of 0.44 M sucrose and recentrifuging for 10 min. The combined supernatants were centrifuged at 17,300 g[1] for 15 min to sediment a homogeneous, brown mitochondrial fraction. After washing the mitochondrial pellet twice, the combined supernatants were centrifuged at 97,288 g[2] for 1 hr to sediment the red, jellylike, microsomal fraction. The final supernatant constituted the last fraction. All equipment was precooled and the centrifuges were maintained at approximately 0 to 2°C. The supernatants were separated from the pellets in the cold room by withdrawing the soluble phase with glass syringes fitted with blunt needles. To facilitate separation, the clear, cellulose nitrate centrifuge tubes were mounted near eye level and illuminated from behind.

None of the initial homogenate was discarded at any step of the fractionation procedure. Each particulate pellet was resuspended in a volume of 0.44 M sucrose that was equal to the initial volume of the unfractionated homogenate so that the amount of particulate material in 1 ml of a resuspended pellet was equivalent to the amount of that same particulate material in 1 ml of unfractionated homogenate. Since combining the supernatants from pellet washing resulted in a 3-fold dilution of the final supernatant, 3 ml of this fraction contained the soluble substances originally present in 1 ml of unfractionated homogenate.

The fractionation method employed was essentially a modified Hogeboom et al. (1948) procedure. Appropriate centrifugal forces were determined for the shell gland homogenate suspended in 0.44 M sucrose by monitoring each fraction for cytochrome oxidase (Cooperstein and Lazarow, 1951) and by observing pellet sections (Palade and Siekevitz, 1956) with the electron microscope as shown in Fig. 1. Since cytochrome oxidase is known to be associated with the mitochondria (Schneider, 1946; Hogeboom et al., 1946), the centrifugal speeds were adjusted until the enzyme activity was concentrated mainly in the mitochondrial fraction. The nuclear fraction consisted of all particulate material that was more dense than the cytochrome oxidase-containing fraction, and the microsomal fraction included the particulate material that was less dense. The mean cytochrome oxidase activities[3] for the total homogenate, nuclear, mitochondrial, microsomal, and supernatant fractions of the first

[1] Servall RC-2 centrifuge, rotor SS-34, r_{max} = 4.25 in.

[2] Spinco Model L Ultracentrifuge, rotor 50, r_{max} = 7.1 cm.

[3] Δlog (ferrocytochrome c) per minute for a 1:100 tissue dilution (Cooperstein and Lazarow, 1951).

four shell gland preparations were 5.83, 0.88, 3.11, 0.69, and 0.08, respectively. The mitochondrial fraction clearly showed the highest activity. There was essentially no activity in the supernatant fraction, and the activity in the nuclear and microsomal fractions was attributed to mitochondrial contamination.

Figure 1 *a* shows the low speed pellet that contained nuclei which were both free and within ruptured cells. Nuclei were not observed in any other fraction. In addition to the structures shown in Fig. 1 *a*, there were aggregates of cellular material that appeared to have been from partially homogenized cells, some whole or nearly intact cells, cilia, and various granules and portions of membranes. The presence of mitochondria (usually in aggregates of partially homogenized material) accounted for the cytochrome oxidase activity in this fraction. Figure 1 *b* is a typical electron micrograph of the mitochondrial fraction, demonstrating the predominance of free mitochondria. Although the mitochondria are pleomorphic, there appears to have been a condensation of the mitochondrial matrix of most mitochondria due to the isolation procedure. Figure 1 *c* shows the microsomal fraction which demonstrates the membranous vesicular material typical of this fraction. No intact or readily recognizable mitochondria were observed in this fraction although some cytochrome oxidase activity was detected here. It is noteworthy that neither the centrifugation forces required, the gross appearance of the pellets, nor the low-magnification electron micrographs revealed any strikingly unique features about the manner in which the shell gland homogenates sedimented as compared with other commonly studied tissues.

RADIOACTIVE DETERMINATIONS: Aliquots of 9 ml from the supernatants and 3 ml aliquots from all other fractions and homogenates were pipetted into porcelain crucibles, dried overnight at 100°C, and ashed 8 to 10 hr at 600°C in a muffle furnace to remove the sucrose and all organic material. To prevent spattering of the sucrose at its melting point, the samples were precharred 1 to 2 hr at 180 to 200°C in an oven with a more controllable temperature. The ashed material was brought into solution with 5% nitric acid, quantitatively transferred to stainless steel, 1.25 in., concentric ring planchets, dried, and counted with an automatic counting device in the Geiger region. The Ca^{45} recovery from fractionation relative to the total homogenate was usually 100 ± 5%.

NITROGEN DETERMINATIONS: Samples of the homogenates and fractions which contained 0.2 to 1.0 mg of nitrogen were analyzed by the micro-Kjeldahl method according to Ma and Zuazaga (1942). The total homogenates contained 1.5 to 2.1 mg of nitrogen per milliliter of sample, and recoveries from fractionation relative to the total homogenates were 100 ± 5%. The average nitrogen content in nine experiments, expressed as percentage of total homogenate, was 29% in the nuclear fraction, 11% in the mitochondrial fraction, 15% in the microsomal fraction, and 43% in the supernatant fraction.

The amount of Ca^{45}, in counts per minute, in the subcellular fractions is expressed as a relative concentration based on nitrogen content, the final expression being counts per minute Ca^{45} per milligram of nitrogen (CPM Ca^{45}/mg N).

Preparation and Analysis of the Liver

In most experiments, the liver was removed in addition to the shell gland. It was chilled and diced, and a portion of wet tissue was weighed out equal to the wet weight of the shell gland mucosal cells. The remainder of the treatment was identical with that used for the shell gland. The per cent calcium and nitrogen recoveries were of the same order as those from the shell gland, and the average nitrogen content in six experiments was approximately 13% in the nuclear fraction, 18% in the mitochondrial fraction, 24% in the microsomal fraction, and 44% in the supernatant fraction. The cytochrome oxidase assays and electron micrographs showed the same general pattern for the liver subcellular fractions as for the shell gland fractions. The liver fractions, however, were not so cleanly separated according to the criteria used.

RESULTS

Short Term Experiments

The distribution of CPM Ca^{45}/mg N in the homogenates and subcellular fractions of the shell gland and the liver for each short term experiment is shown in Table I. The mean values and the standard errors were calculated from these data and are presented in Fig. 2. In both tissues, essentially all the Ca^{45} was associated with the particulate fractions; the supernatant fractions were almost devoid of Ca^{45} even though they contained approximately 45% of the cellular nitrogen. In spite of the large variability from experiment to experiment in the amount of CPM Ca^{45}/mg N that was detected in the cells, the mean values show significant differences among the fractions in both the shell gland and the liver. In the shell gland, the mean CPM Ca^{45}/mg N value of the mitochondrial fraction is significantly higher than that of the nuclear fraction ($P < 0.01$); and the mean value of the nuclear fraction is significantly higher than that of the microsomal fraction ($P < 0.01$). For the corresponding liver fractions, the differ-

TABLE I

Relative Concentration of Calcium^{45} in Each Subcellular Fraction for Each Short Term Experiment

Experiment	1	2	3	4	5	6	7	8	9
					CPM Ca^{45}/mg N				
Shell gland									
Total homogenate	730	1153	704	537	667	365	349	374	872
Nuclear fraction	1058	1794	1168	904	1198	600	553	592	1408
Mitochondrial fraction	1127	3917	2037	1331	1755	1272	1191	1043	2962
Microsomal fraction	623	417	556	674	261	159	254	194	611
Supernatant fraction	117	124	87	114	87	56	80	84	213
Liver									
Total homogenate	—	—	378	354	—	215	308	286	386
Nuclear fraction	—	—	727	701	—	334	516	549	590
Mitochondrial fraction	—	—	649	669	—	493	960	829	1182
Microsomal fraction	—	—	361	220	—	183	163	122	241
Supernatant fraction	—	—	178	117	—	94	61	36	71

ence is not significant in the former case, although the trend is the same, and is significant ($P < 0.001$) in the latter case. Therefore, both types of cells appear to have the same "qualitative pattern" of CPM Ca^{45}/mg N.

In Table II, the shell gland and liver cells are quantitatively compared by determining the shell gland-to-liver ratios of the means of the homogenates and fractions from Fig. 2. These ratios reveal two points: (1) since all the ratios are greater than 1, the shell gland has a higher value of CPM Ca^{45}/mg N than the liver in the total homogenate and in every fraction; and (2) since the mitochondrial ratio is largest, the shell gland mitochondrial fraction incorporated a disproportionately greater quantity of the Ca^{45} than did the other shell gland fractions and the unfractionated homogenate.

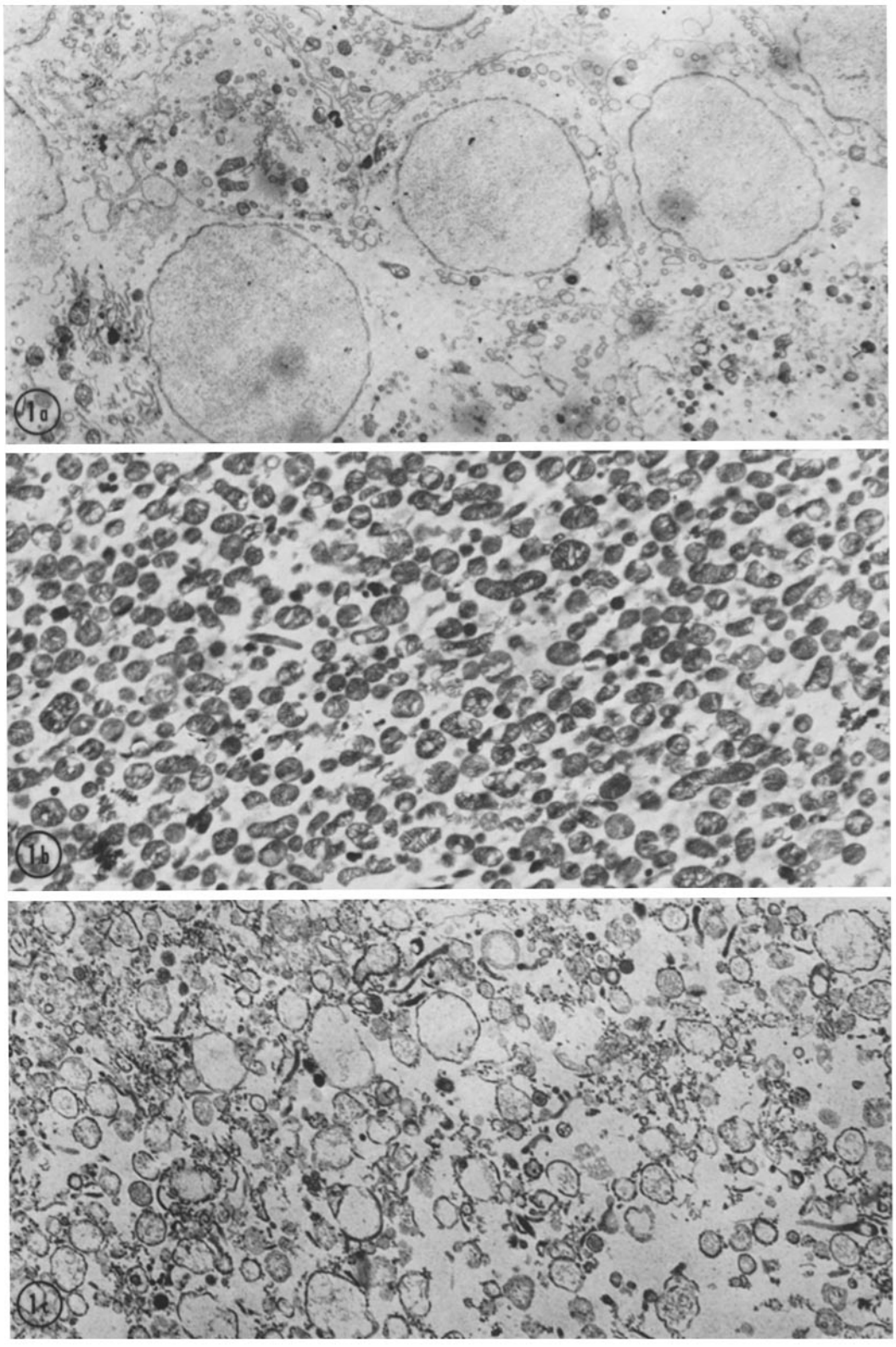
To reduce the variability due to the different amounts of Ca^{45} that were detected in the cells from one experiment to the next (Table I), we used each experiment as its own control by determining the ratio of CPM Ca^{45}/mg N of each fraction to that of the total homogenate. These ratios for each experiment, together with the mean values and standard errors, are presented in Table III. These same values would result from calculating first the per cent Ca^{45} and per cent nitrogen in each fraction and then the ratio of per cent Ca^{45} to per cent nitrogen, according to the method used by Thiers and Vallee (1957). The mitochondrial fraction again contained the largest proportion of CPM Ca^{45}/mg N in both the shell gland and the liver. When the mean values of the shell gland and the liver fractions are compared by determining the shell gland to liver ratios in the manner used in Table II, the ratio is greater than 1 only in the mitochondrial fraction. This result again suggested that the shell gland mitochondrial fraction ac-

FIGURE 1 Survey electron micrographs of each of the three particulate pellets isolated by differential centrifugation.

FIGURE 1 *a* Nuclear fraction (755 *g*). The nuclei, both free and inside ruptured cells, as well as portions of cells, mitochondria, membrane fragments, and granules are evident. (Pellet was recentrifuged at 12,000 *g* to obtain closer packing for electron microscopy.) × 5000.

FIGURE 1 *b* Mitochondrial fraction (17,300 *g*). The predominance of free mitochondria in this pellet is evident. Granules, membranous structures, and cilia may also be seen. × 5000.

FIGURE 1 *c* Microsomal fraction (97,288 *g*). Various sizes of microsomal membranous vesicles dominate this fraction. Membrane fragments, small granules, portions of cilia and ribosomes also are evident. × 15,000.


1a
1b
1c

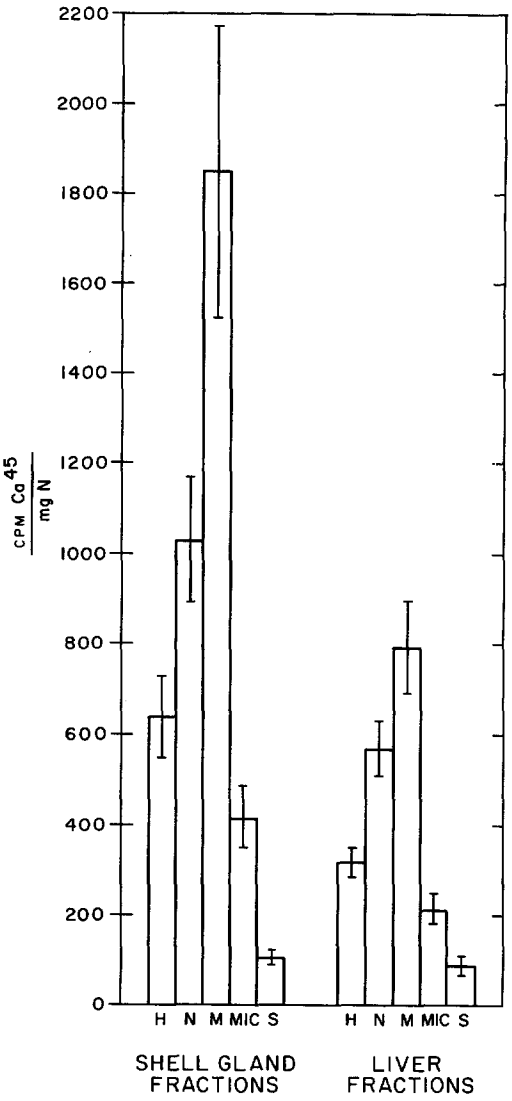


FIGURE 2 The distribution of CPM Ca^{45}/mg N in the subcellular fractions of the shell gland and liver short term experiments. Each bar represents the mean of nine experiments for the shell gland and six experiments for the liver. Standard errors are indicated. *H*, total homogenate; *N*, nuclear fraction; *M*, mitochondrial fraction; *Mic*, microsomal fraction; and *S*, supernatant fraction.

cumulated more than a proportional amount of Ca^{45} as compared with the liver mitochondrial fraction. The nuclear and microsomal ratios (0.92 and 1.00, respectively) show that these fractions appeared to accumulate Ca^{45} in similar proportions in both cell types. The supernatant ratio is much less than 1 (0.59), but the quantity of radioactivity was so low that this ratio does not represent a very large absolute difference between these fractions.

TABLE II

Comparison of Shell Gland and Liver Subcellular Fractions of the Short Term Experiments

Fraction	Shell gland-to-liver*
Total homogenate	1.99
Nuclear fraction	1.81
Mitochondrial fraction	2.32
Microsomal fraction	1.94
Supernatant fraction	1.15

* Ratio of mean CPM Ca^{45}/mg N values from Fig. 2.

In Fig. 2, the standard errors indicate that the shell gland demonstrated considerably more variability from experiment to experiment than the liver. This variability was thought to be due to the state of the physiological activity of the shell gland with respect to the laying cycle in the various experiments. Therefore, the data for the shell gland in Table I were grouped according to three physiological states of the experimental animals at the time of sacrifice: (1) when calcification was not occurring (noncalcifying), i.e. when no egg was present in the oviduct; (2) just prior to calcification (precalcifying), i.e. when an egg was present in the isthmus (the region of the oviduct which precedes the shell gland and is responsible for shell membrane formation); and (3) when calcification was occurring (calcifying), i.e. when an egg was present in the shell gland. The mean values and standard errors in CPM Ca^{45}/mg N for each state are shown in Fig. 3. The CPM Ca^{45}/mg N was always highest in the mitochondrial fraction for each of the three physiological states. When calcification was not occurring (noncalcifying), the CPM Ca^{45}/mg N level appeared to be higher than the other two physiological states in the homogenate and in all fractions, with the notable exception of the microsomal fraction. Here, the CPM Ca^{45}/mg N level was highest when calcification was occurring (calcifying). Two significant points should be noted: (1) Ca^{45} appeared to accumulate in the cells, predominately in the mitochondrial fraction, when calcification was not occurring; and (2) the CPM Ca^{45}/mg N was highest for that microsomal fraction obtained when calcification was occurring, a result which suggested a possible

TABLE III

Ratios of Each Fraction (CPM Ca^{45}/*mg N*) *to the Total Homogenate* (CPM Ca^{45}/*mg N*) *for Each Short Term Experiment*

Experiment	1	2	3	4	5	6	7	8	9	Mean ± SE
Shell gland										
Nuclear-to-homogenate	1.45	1.56	1.66	1.68	1.80	1.64	1.58	1.58	1.61	1.62 ± 0.03
Mitochondrial-to-homogenate	1.54	3.40	2.89	2.48	2.63	3.48	3.41	2.79	3.40	2.89 ± 0.21
Microsomal-to-homogenate	0.85	0.36	0.79	1.26	0.39	0.44	0.73	0.52	0.70	0.67 ± 0.10
Supernatant-to-homogenate	0.16	0.11	0.12	0.21	0.13	0.15	0.23	0.22	0.24	0.17 ± 0.02
Liver										
Nuclear-to-homogenate	—	—	1.92	1.98	—	1.55	1.68	1.92	1.53	1.76 ± 0.08
Mitochondrial-to-homogenate	—	—	1.72	1.89	—	2.29	3.12	2.90	3.06	2.50 ± 0.25
Microsomal-to-homogenate	—	—	0.96	0.62	—	0.85	0.53	0.43	0.62	0.67 ± 0.08
Supernatant-to-homogenate	—	—	0.47	0.33	—	0.44	0.20	0.13	0.18	0.29 ± 0.06

relationship of Ca^{45} with this subcellular fraction at this time. Just prior to calcification, when an egg was in the isthmus, the mean values for each fraction tended to be slightly lower than those of the other two physiological states.

The data for the shell gland fractions in Table III were also regrouped according to the three physiological states. The mean ratios of each fraction for each state are presented in Table IV. The mitochondrial ratios again are shown to be highest from among the other fractions in all three states. To compare relative Ca^{45} contents according to the physiological state, we calculated the ratios of the noncalcifying-to-calcifying states for each fraction and included them in Table IV. The mitochondrial-to-homogenate ratio is decidedly higher when calcification was not occurring than when it was occurring, with a comparative ratio of 1.50. The microsomal-to-homogenate ratios also are altered in accordance with the physiological activity; the mean value is half as high when calcification was not occurring as when it was occurring, with a comparative ratio of 0.53. Since the comparative ratio for the microsomal fraction is less than 1.00 (0.53) and that for the mitochondrial fraction is greater than 1.00 (1.50), a nonparallel participation of these two subcellular fractions is evident. In contrast to the mitochondrial-to-homogenate and microsomal-to-homogenate ratios, the nuclear-to-homogenate and supernatant-to-homogenate ratios are relatively constant regardless of the physiological activity. The noncalcifying-to-calcifying value was 1.00 in both cases.

When the egg was in the isthmus (precalcifying) the activity of the mitochondrial fraction was intermediate between the other two physiological states, and that of the microsomal fraction was approximately the same as the noncalcifying state.

Long Term Experiments

In view of the 5 min Ca^{45} exposure time used in the above experiments, we determined the distribution of CPM Ca^{45}/mg N in shell gland mucosa and liver cells after prolonged exposure to Ca^{45}. Although the results of two experiments are preliminary, they strongly support the short term experiments and, therefore, are presented. In Experiment A, the shell gland was in a noncalcifying state since the egg was in the magnum (the region of the oviduct where the albumin is secreted), and the hen received the last Ca^{45} injection approximately 2 hr before sacrifice. In Experiment B, the shell gland was in a calcifying state since the egg was in the shell gland, and the hen received the last Ca^{45} injection approximately 24 hr before sacrifice. Since only two long term experiments were performed and the net CPM Ca^{45} in some fractions was only slightly above background, numerical comparisons parallel to those made for the short term experiments were not practical and only qualitative comparisons are noted.

The relative concentrations of Ca^{45} in the subcellular fractions for the long term experiments are presented in Table V. The two types of long term experiments may be compared to the short term experiments with respect to (1) the over-all

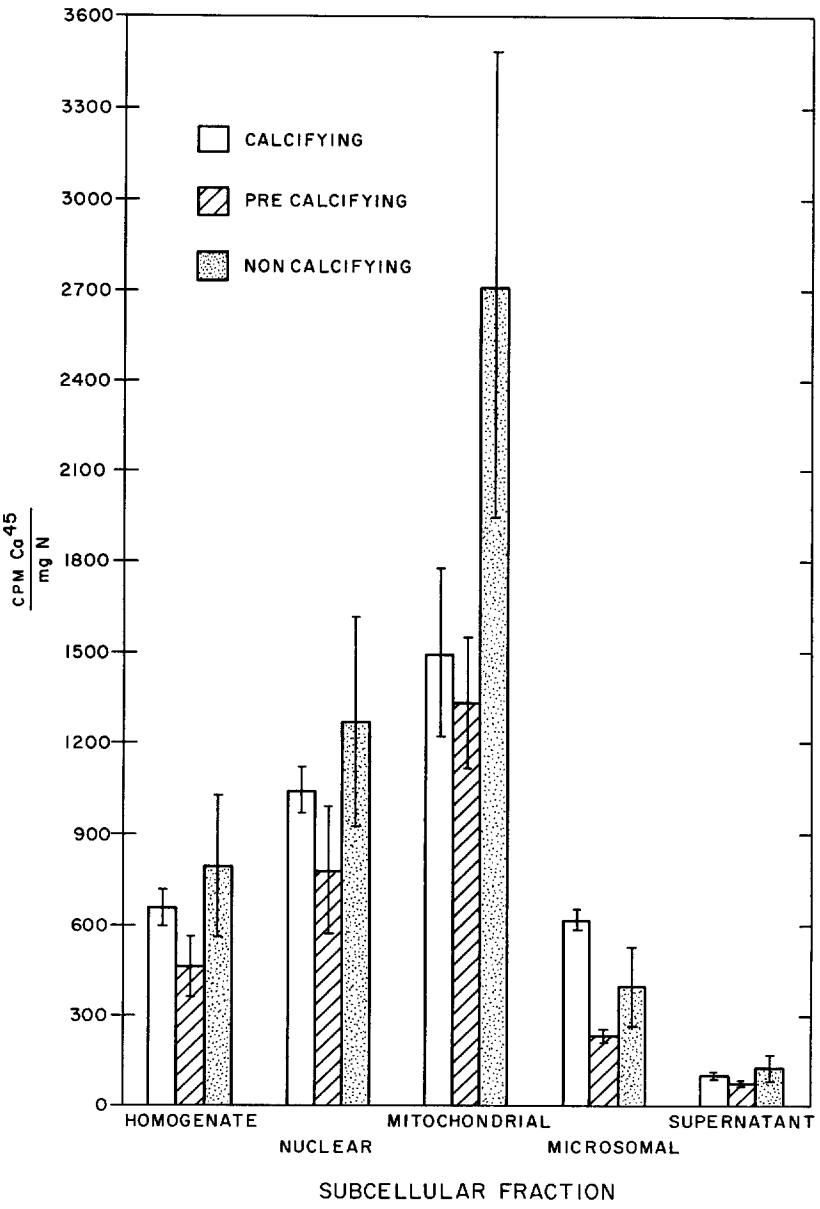


FIGURE 3 The distribution of CPM Ca^{45}/mg N in the subcellular fractions of the shell gland short term experiments according to the physiological activity at the time of sacrifice. Each bar represents the mean of three experiments. Standard errors are indicated.

CPM Ca^{45}/mg N distribution, (2) the differences between the shell gland and liver cells, (3) the effects of the elapsed time between injection and sacrifice, and (4) the changes which occurred owing to the physiological activity at the time of sacrifice. Since more than one variable was changed in the experimental conditions, the effects overlap one another. However, the differences are of sufficient magnitude to permit noting one effect at a time:

1. As in the short term experiments, the Ca^{45} of both long term experiments was associated with

TABLE IV

Comparison of Mean Ratios of Fraction (CPM Ca^{45}/mg N) to Homogenate (CPM Ca^{45}/mg N) According to the Physiological State of the Shell Gland for the Short Term Experiments

Physiological state*	Noncalcifying	Precalcifying	Calcifying	Noncalcifying-to-calcifying
Nuclear-to-homogenate	1.59	1.69	1.59	1.00
Mitochondrial-to-homogenate	3.41	2.87	2.28	1.50
Microsomal-to-homogenate	0.50	0.51	0.94	0.53
Supernatant-to-homogenate	0.16	0.18	0.16	1.00

* N, 3 for each physiological state.

TABLE V

Relative Concentration of Calcium^{45} in Each Subcellular Fraction for Each Long Term Experiment

Experiment Time from last injection	A 2 hr	B 24 hr
	CPM Ca^{45}/mg N	
Shell gland		
Total homogenate	212	82
Nuclear fraction	379	172
Mitochondrial fraction	483	120
Microsomal fraction	58	74
Supernatant fraction	18	6
Liver		
Total homogenate	60	14
Nuclear fraction	58	14
Mitochondrial fraction	58	22
Microsomal fraction	50	9
Supernatant fraction	22	4

the particulate fractions in both shell gland and liver, and not bound to the soluble substances present in the supernatant fractions.

2. Also, as in the short term experiments, the shell gland homogenates and fractions of both long term experiments contained more CPM Ca^{45}/mg N than their corresponding liver homogenates and fractions.

3. Comparison of the effects of the elapsed time between the last injection and sacrifice is shown in Fig. 4 where the CPM Ca^{45}/mg N of only the homogenates of the shell gland and liver are shown for the three labeling periods. As the elapsed time increased, the CPM Ca^{45}/mg N present in the cells of both tissues greatly decreased. Furthermore, the shell gland Ca^{45} concentration decreased more slowly than the liver Ca^{45} concentration. This may be observed in two ways. First, the relative differences between the shell gland homogenate and the liver homogenate became larger as the elapsed time increased; the ratios of shell gland homogenate to liver homogenate were approximately 2 in the 5 min experiments, 3.5 in the 2 hr experiment, and 5.9 in the 24 hr experiment. Second, between the 5 min and 2 hr labeling periods, the liver CPM Ca^{45}/mg N decreased about 81%, whereas the corresponding decrease was only about 67% in the shell gland. Likewise, between the 2 hr and the 24 hr labeling periods, the liver CPM Ca^{45}/mg N decreased about 77%, whereas the corresponding decrease was only 61% in the shell gland.

4. Finally, the changes which occur due to the physiological activity of the shell gland may be seen in Table VI for the long term experiments where the data from Table V were used to calculate the proportion of CPM Ca^{45}/mg N in each fraction according to the manner used in Table IV for the short term experiments. Also, as in Table IV, the noncalcifying and calcifying states are compared by calculating the ratio of the two states for each fraction. As in the short term experiments, the mitochondrial-to-homogenate ratio is higher when calclification was not occurring (Experiment A) than when calcification was occurring (Experiment B), the comparative ratio was 1.56. When calcification was occurring, the mitochondrial CPM Ca^{45}/mg N level was so low that it fell below the nuclear level (Experiment B, Tables V, VI). The A-to-B ratio of 0.30 for the microsomal fractions indicates that there was relatively less Ca^{45} in this fraction when calcification was not occurring than when it was occurring. Again the mitochondrial and microsomal fractions are shown to participate in a nonparallel manner as already noted in the results of the short term experiments. Also, in agreement with the results of the short term experiments, the nuclear- and

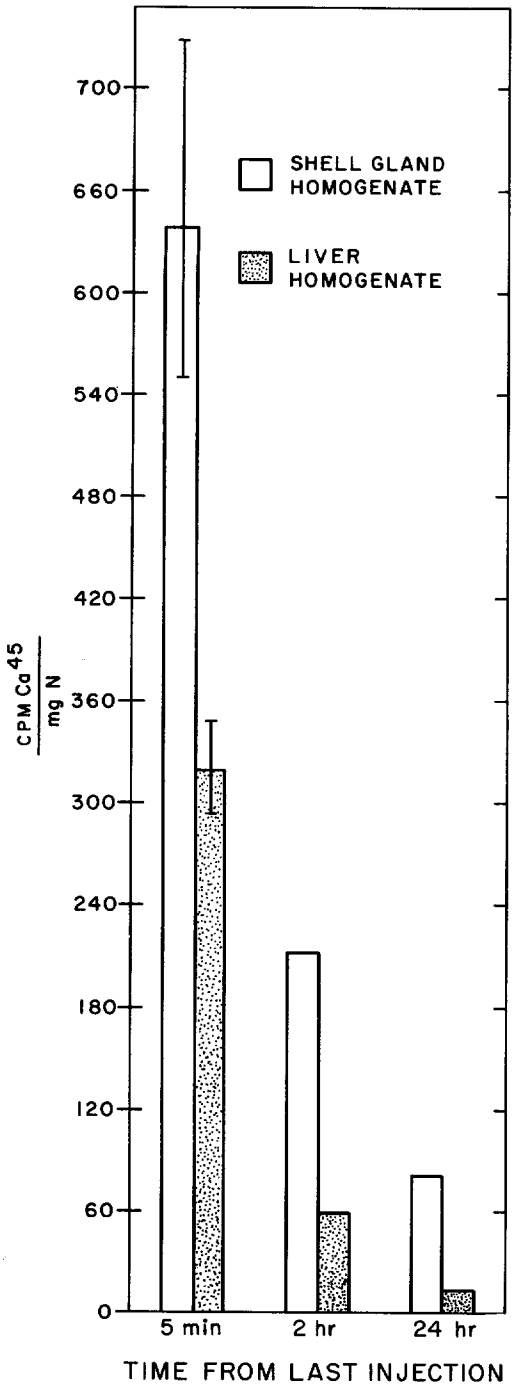


FIGURE 4 The distribution of CPM Ca^{45}/mg N in the homogenates of the shell gland and liver according to the time elapsed since the last injection of Ca^{45}. Each bar of the 5 min (short term) experiments represents the mean of 9 experiments for the shell gland and 6 experiments for the liver. Standard errors are indicated. Each bar of the 2 hr and 24 hr (long term) experiments represents one experiment.

supernatant-to-homogenate ratios are relatively constant, indicating that the proportion of CPM Ca^{45}/mg N in these two fractions did not appear to change as a function of the physiological activity.

DISCUSSION

Two features which make the shell gland an excellent system for the study of calcium movement through cells are: (1) relatively large quantities of calcium are transported in a discrete period of time; and (2) the cellular calcium transport system is separated from the external site of calcium deposition, permitting investigation of transport phenomena without interference by final intrinsic deposition of calcium.

Several observations indicated that the in vivo, 5 min, labeling period in the short term experiments appeared to be adequate for transport and distribution studies. Well labeled egg shells provided proof that calcium transport was occurring at the time of sacrifice. Preliminary experiments showed that a shorter labeling period of 2 min and a more indirect route of Ca^{45} administration by intramuscular injection still yielded well labeled shells. Longer labeling periods (30 days) yielded results which complemented those of the 5 min labeling period. The qualitative Ca^{45} distribution pattern reported here is similar to that obtained in other tissues.

Nitrogen content was employed as a standard unit of reference for expressing the Ca^{45} activity in the subcellular fractions. The problems involved in choosing an appropriate reference have been discussed by Thiers and Vallee (1957), who stated, "The expression of metal concentrations in tissue is rendered difficult by lack of any base line with an inherent justification." They showed that different distributions resulted from using nitrogen and ash weights as base lines. The amount of nitrogen per unit weight or volume may not be uniformly distributed among the different subcellular fractions, and the amount of nitrogenous substances may not be constant for each physiological state of the cells when they are harvested. These problems still remain.

The general pattern of Ca^{45} distribution observed among the intracellular fractions of the shell

gland mucosal cells (Fig. 2) is qualitatively similar to the calcium distributions of other tissues which have been investigated (Griswold and Pace, 1956; Thiers and Vallee, 1957; Cosmos, 1964; Cassidy et al., 1965). The distribution of Ca^{45} in the hen liver intracellular fractions also showed a similar qualitative pattern (Fig. 2). In these studies calcium was observed to be associated mainly with the particulate subcellular components rather than with the soluble substances; the mitochondrial fraction was generally the principal calcium acceptor. Therefore, the subcellular organelles which accumulated the highest CPM Ca^{45}/mg N relatively high CPM Ca^{45}/mg N levels in the shell gland may be indicative of its specialized ion transport function.

It is clear from Fig. 4 that there was no permanent storage or accumulation of calcium in either the shell gland or liver cells. The initial rapid accumulation of Ca^{45} in the cells immediately after injection was always followed by loss of Ca^{45} as time progressed.

Except for small differences when the egg was in the isthmus, the results reported here agree in general with the study which showed that the amount of calcium in the shell gland tissue is a

TABLE VI

Comparison of Ratios of Fraction (CPM Ca^{45}/mg N) to Homogenate (CPM Ca^{45}/mg N) According to the Physiological State of the Shell Gland for the Long Term Experiments

Physiological state	Noncalcifying (Exp. A)	Calcifying (Exp. B)	Ratio (A-to-B)
Nuclear-to-homogenate	1.79	2.10	0.85
Mitochondrial-to-homogenate	2.28	1.46	1.56
Microsomal-to-homogenate	0.27	0.90	0.30
Supernatant-to-homogenate	0.08	0.07	1.14

levels in the shell gland appeared to be the same as those which accumulated calcium in the other tissues investigated.

In comparison with the liver cells, which are not specialized for ion transport, the shell gland cells incorporated larger quantities of CPM Ca^{45}/mg N in both the short and long term experiments (Fig. 4). Since both tissues are well supplied with blood, and the Ca^{45} was introduced directly into the circulatory system of the experimental animal, both the shell gland and the liver were initially exposed to a relatively large dose of Ca^{45}. In the short term experiments, it is likely that this initial high Ca^{45} level tended to mask somewhat the selectivity for calcium that the shell gland appeared to have with respect to the liver (Table II). With the passage of time, this greater affinity of the shell gland for Ca^{45} relative to the liver became much more evident (Fig. 4, Table V). As can be seen in Table V, the net CPM Ca^{45}/mg N for the liver was already low in the 2 hr experiment (A) and was barely measurable in the 24 hr experiment (B), whereas the Ca^{45} activity was still readily detectable in the shell gland particulate fractions in both cases. This observation suggests that the low CPM Ca^{45}/mg N levels in the liver may only reflect the availability of Ca^{45} in the serum, whereas the function of its physiological state (Schraer and Schraer, 1965).

The Mitochondrial Fraction

The mitochondrial fraction of both the shell gland and the liver appeared to be one of the intracellular sites which possesses an affinity for calcium (Figs. 2 and 3; Tables I to VI). The results reported here and those of other investigators show the affinity of mitochondria for calcium in living animals. In vitro binding of calcium to mitochondria also has been observed (Slater and Cleland, 1953; DeLuca and Engstrom, 1961; Vasington and Murphy, 1962; Lehninger et al., 1963; Brierley et al., 1963; Engstrom and DeLuca, 1964). Digitonin-treated mitochondria bind calcium (Vasington, 1963); vitamin D and parathyroid hormone influence mitochondrial calcium binding (DeLuca et al., 1962; Rasmussen and DeLuca, 1963); and carbon tetrachloride (Thiers et al., 1960) and thioacetamide (Gallagher et al., 1956) cause mitochondrial calcium to increase markedly.

The following observations between calcium and the shell gland mitochondrial fraction suggest a functional role for the mitochondria in this system: (1) The shell gland mitochondrial fraction had a greater affinity for calcium than the liver

mitochondrial fraction (Fig. 2; Tables II, III, and V). (2) Ca^{45} was preferentially incorporated by the mitochondrial fraction in the presence of all other cell structures (Figs. 2 and 3; Tables I to VI). (3) The uptake of Ca^{45} by the mitochondrial fraction, even when transport had been occurring for only 5 min, was immediate and rapid (Fig. 2; Tables I and III). (4) When shell calcification was not occurring, the largest proportion of the Ca^{45} which accumulated in the mucosal cells was located in the mitochondrial fraction (Fig. 3; Tables IV and VI). (5) When shell calcification was occurring, the quantity of CPM Ca^{45}/mg N in the mitochondrial fraction was much lower than when calcification was not occurring (Fig. 3; Tables IV and VI). This depressed Ca^{45} level of the mitochondrial fraction when calcification was occurring suggested that the mitochondria may have yielded Ca^{45} to the deposition site. No one of these reasons is proof that mitochondria had an active role in the transport process, but when considered together they indicate that passive accumulation was unlikely. We still lack conclusive proof that the Ca^{45} detected in the mitochondria was destined to be transported to the deposition site and that the calcium must go to the mitochondria in traversing the cell.

Other investigators also have suggested a functional role for the mitochondria in the transcellular movement of ions (Thiers et al., 1960; Vasington and Murphy, 1962; Cosmos, 1964; Peachey, 1964). Recent work in which calcium transport across in vitro preparations of intact shell gland tissue was studied showed that this process may be linked with the metabolic functions of the mitochondria (Schraer et al., 1965). Not only has a functional role of calcium control been suggested for the mitochondria, but also structural studies have demonstrated possible binding sites for calcium in the form of granules within the mitochondria (Gonzales and Karnovsky, 1961; Brierley and Slautterback, 1964; Greenawalt et al., 1964; Peachey, 1964; Reynolds, 1965; Weinbach and von Brand, 1965). Thus, the evidence from these experiments and the work of others strongly suggest that the mitochondria of the shell gland participate in the movement of calcium.

The Microsomal Fraction

When the physiological state of the experimental animal was considered, the relative Ca^{45} level in the microsomal fraction appeared to be altered in both the short term (Fig. 3) and long term experiments. When calcification was occurring, the microsomal CPM Ca^{45}/mg N was almost equal to that of the total homogenate, the microsomal fraction to homogenate ratios were 0.94 (Table IV) and 0.90 (Table VI). When calcification was not occurring, the microsomal values were much less than the homogenate values; the ratios were 0.50 (Table IV) and 0.27 (Table VI). It is possible that the Ca^{45} was "trapped" in this fraction while *en route* either to the calcification site in the lumen of the shell gland or to (or from) the mitochondria. Whether the association of calcium with this fraction was passive, i.e. trapped in its path through the cell, or active, i.e. possessing physical or biochemical dependencies, was not answered by these experiments. Energy-dependent calcium accumulation in muscle microsomal fractions has been demonstrated (Ebashi and Lipmann, 1962; Molnar and Lorand, 1962), and the endoplasmic reticulum has been previously postulated as a mediator for the transport of calcium to and from the mitochondria (Cosmos, 1964).

The Nuclear Fraction

The comparison of the mean shell gland nuclear fraction with that of the liver (Table II), the mean ratio of the nuclear fractions to their homogenates of both tissues (Table III), and the lack of response to the physiological activity of the tissue or to the time elapsed from the last Ca^{45} injection (Tables IV and VI) showed that the nuclear fraction appeared to have accumulated Ca^{45} passively and did not seem to participate in the transport process. Since the nuclear fraction contained a large variety of cellular substructures, it is difficult to assign the Ca^{45} activity of this fraction to specific components. Nuclei have been shown to contain calcium in several types of cells (Mirsky, 1961). Contamination of this fraction by mitochondria, partially ruptured cells, and aggregates of cellular components was likely to have contributed a significant portion of the Ca^{45} activity. Whether or not calcium was associated with cellular components such as the cell membranes is not known.

The Supernatant Fraction

The final supernatant fraction was almost devoid of Ca^{45} in spite of containing approximately 45%

of the total nitrogen (Figs. 2 and 3; Tables I, III, and V). Like the nuclear fraction, this fraction did not show any responses which would indicate a role in the calcium transport mechanism (Tables II to IV, and VI).

When the egg was in the isthmus, no consistent results emerged (Fig. 3; Tables IV and VI). In this state the physiological activity of the shell gland may have been in a transitory condition in preparation for commencement of calcification.

It has been assumed in this report that the Ca^{45} remained bound to the subcellular sites at which it accumulated in vivo. Ionic exchange among the subcellular organelles or selective binding of calcium that may have been ionic in vivo, could have occurred during the isolation process to distort the actual distribution. However, two features may have contributed to the validity of our assumptions. The first was that the metabolic activity of the tissue was minimized by immediate chilling and performing all homogenization and fractionation procedures in the cold. Secondly, indirect evidence from biochemical studies on the effect of sucrose on mitochondria indicates that high sucrose concentrations could possibly aid in the maintenance of the initial *status quo* of the calcium distribution. Lehninger et al. (1959) suggested, "The apparent suitability of sucrose for isolation of mitochondria . . . is the result of a reversible inhibition or 'fixation' of an enzyme step in the coupling of respiration with membrane contractility and permeability." Further, Lynn et al. (1964) suggested that, since high concentrations of sucrose (less than the 0.44 M solution used in this report) at 23 to 25°C inhibited both osmotic and metabolic contractions of mitochondria, "The mode of action of sucrose must be to alter the semipermeable property of the membranes," and, therefore, stabilizes or "freezes" the mitochondrial membranes so that they no longer respond to osmotic changes. The low temperature and sucrose medium used here may maintain the mitochondrial calcium content and perhaps be beneficial to the study of ionic distributions in general. Experiments designed to examine the possibility of interorganelle exchange and to determine whether the Ca^{45} distribution occurred in vivo or during the environmental alterations of the experimental procedure have been initiated in our laboratory.

On the basis of this study, the Ca^{45} distributed via the circulatory system was maintained at a higher level by the shell gland cells than by the liver cells. The shell gland mitochondrial fraction appeared to acquire a larger proportion of the Ca^{45} than the liver mitochondrial fraction did, to accumulate Ca^{45} when calcification was not occurring, and to yield Ca^{45} when calcification was occurring. The changes in Ca^{45} levels of the microsomal fraction were opposite to those of the mitochondrial fraction, i.e. the microsomes contained relatively small amounts of Ca^{45} when calcification was not occurring and larger amounts when calcification was occurring. Therefore, Ca^{45} may be temporarily taken up by the mitochondria when calcification is not occurring and passed to the deposition site (egg shell) via the mitochondria and some component of the microsomal fraction when calcification is occurring. Since large Ca^{45} deposits were not accrued in either the shell gland or the liver cells as the time from Ca^{45} injection progressed, a large portion of the initial high Ca^{45} concentrations leaves the cells regardless of whether calcification is or is not occurring. These results show that the ability of the shell gland to sequester and control the movement of calcium is amplified when compared with the liver. Although these suggestions must be supported by additional evidence, it appears that calcium in cells may be regulated by the mitochondria, and that the shell gland may have adapted a normal cellular function to perform its specialized role of transporting large quantities of calcium.

The authors wish to express their appreciation to Dr. Rosemary Schraer, Department of Biochemistry, The Pennsylvania State University, for advice and consultation throughout this study, and to acknowledge the technical assistance of Mrs. Bridget Stemberger and Mrs. Jacqueline Frey.

This study was supported by Research Grant DE 01764, National Institutes of Health.

Received for publication 25 February 1966.

REFERENCES

BRIERLEY, G. P., MURER, E., and GREEN, D. E., 1963, Participation of an intermediate of oxidative phosphorylation in ion accumulation by mitochondria, *Science*, **140**, 60.

BRIERLEY, G. P., and SLAUTTERBACK, D. B., 1964, Studies on ion transport. IV. An electron microscope study of the accumulation of Ca^{++} and inorganic phosphate by heart mitochondria, *Biochim. et Biophysica Acta*, **82**, 183.

CASSIDY, M. M., GOLDNER, A. M., and TIDBALL,

C. S., 1965, Studies on the localization of magnesium and calcium ions in mucosal tissue from canine small intestine, *Fed. Proc.*, **24**, 588(abstract).

COOPERSTEIN, S. J., and LAZAROW, A., 1951, A microspectrophotometric method for the determination of cytochrome oxidase, *J. Biol. Chem.*, **189**, 665.

COSMOS, E., 1964, Intracellular distribution of calcium in developing breast muscle of normal and dystrophic chickens, *J. Cell Biol.*, **23**, 241.

DELUCA, H. F., and ENGSTROM, G. W., 1961, Calcium uptake by rat kidney mitochondria, *Proc. Nat. Acad. Sc.*, **47**, 1744.

DELUCA, H. F., ENGSTROM, G. W., and RASMUSSEN, H., 1962, The action of vitamin D and parathyroid hormone *in vitro* on calcium uptake and release by kidney mitochondria, *Proc. Nat. Acad. Sc.*, **48**, 1604.

EBASHI, S., and LIPMANN, F., 1962, Adenosine triphosphate–linked concentration of calcium ions in a particulate fraction of rabbit muscle, *J. Cell Biol.*, **14**, 389.

ENGSTROM, G. W., and DELUCA, H. F., 1964, The nature of Ca^{++} binding by kidney mitochondria, *Biochemistry*, **3**, 379.

GALLAGHER, C. H., GUPTA, D. N., JUDAH, J. D., and REES, K. R., 1956, Biochemical changes in liver in acute thioacetamide intoxication, *J. Path. Bact.*, **72**, 193.

GONZALES, F., and KARNOVSKY, M. J., 1961, Electron microscopy of osteoclasts in healing fractures of rat bone, *J. Biophysic. and Biochem. Cytol.*, **9**, 299.

GREENAWALT, J. W., ROSSI, C. S., and LEHNINGER, A. L., 1964, Effect of active accumulation of calcium and phosphate ions on the structure of rat liver mitochondria, *J. Cell Biol.*, **23**, 21.

GRISWOLD, R. L., and PACE, N., 1956, The intracellular distribution of metal ions in rat liver, *Exp. Cell Research*, **11**, 362.

HERTELENDY, F., and TAYLOR, T. G., 1961, Changes in blood calcium associated with egg shell calcification in the domestic fowl. I. Changes in the total calcium, *Poultry Sc.*, **40**, 108.

HOGEBOOM, G. H., CLAUDE, A., and HOTCHKISS, R. D., 1946, The distribution of cytochrome oxidase and succinoxidase in the cytoplasm of the mammalian liver cell, *J. Biol. Chem.*, **165**, 615.

HOGEBOOM, G. H., SCHNEIDER, W. C., and PALADE, G. E., 1948, Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material, *J. Biol. Chem.*, **172**, 619.

LEHNINGER, A. L., RAY, B. L., and SCHNEIDER, M., 1959, The swelling of rat liver mitochondria by thyroxine and its reversal, *J. Biophysic. and Biochem. Cytol.*, **5**, 97.

LEHNINGER, A. L., ROSSI, C. S., and GREENAWALT, J. W., 1963, Respiration-dependent accumulation of inorganic phosphate and Ca^{++} by rat liver mitochondria, *Biochem. and Biophysic. Research Commun.*, **10**, 444.

LYNN, W. S., FORTNEY, S., and BROWN, R. H., 1964, Osmotic and metabolic alterations of mitochondrial size, *J. Cell Biol.*, **23**, 1.

MA, T. S., and ZUAZAGA, G., 1942, Micro-Kjeldahl determination, *Ind. and Eng. Chem., Analytical Edition*, **14**, 280.

MIRSKY, A. E., and OSAWA, S., 1961, The interphase nucleus, *in* The Cell (J. Brachet and A. E. Mirsky, editors), Academic Press Inc., New York, **2**, 737.

MOLNAR, J., and LORAND, L., 1962, A phosphoryl group acceptor attached to the microsomal fraction of muscle, *Arch. Biochem. and Biophysics*, **98**, 356.

PALADE, G. E., and SIEKEVITZ, P., 1956, Liver microsomes. An integrated morphological and biochemical study, *J. Biophysic. and Biochem. Cytol.*, **2**, 171.

PEACHEY, L. D., 1964, Electron microscopic observations on the accumulation of divalent cations in intramitochondrial granules, *J. Cell Biol.*, **20**, 95.

POTTER, V. R., and ELVEHJEM, C. A., 1936, A modified method for the study of tissue oxidations, *J. Biol. Chem.*, **114**, 495.

RASMUSSEN, H., and DELUCA, H. F., 1963, Calcium homeostasis, *Ergebn. Physiol. Biol. Chem. u. Exp. Pharmakol.*, **53**, 108.

REYNOLDS, E. S., 1965, Liver parenchymal cell injury. III. The nature of calcium-associated electron-opaque masses in rat liver mitochondria following poisoning with carbon tetrachloride, *J. Cell Biol.*, **25**, 53.

SCHNEIDER, W. C., 1946, Intracellular distribution of enzymes. I. The distribution of succinic dehydrogenase, cytochrome oxidase, adenosine triphosphatase, and phosphorus compounds in normal rat tissues, *J. Biol. Chem.*, **165**, 585.

SCHRAER, H., HOHMAN, W., EHRENSPECK, G., and SCHRAER, R., 1965, Subcellular distribution of calcium in the avian shell gland in relation to calcium transport, *J. Cell Biol.*, **27**, 96A(abstract).

SCHRAER, R., and SCHRAER, H., 1965, Changes in metal distribution of the avian oviduct during the ovulation cycle, *Proc. Soc. Exp. Biol. and Med.*, **119**, 937.

SLATER, E. C., and CLELAND, K. W., 1953, The effect of calcium on the respiratory and phosphorylative activities of heart-muscle sarcosomes, *Biochem. J.*, **55**, 566.

THIERS, R. E., REYNOLDS, E. S., and VALLEE, B. L., 1960, The effect of carbon tetrachloride poison-

ing on subcellular metal distribution in rat liver, *J. Biol. Chem.*, **235**, 2130.

THIERS, R. E., and VALLEE, B. L., 1957, Distribution of metals in subcellular fractions of rat liver, *J. Biol. Chem.*, **226**, 911.

VASINGTON, F. D., 1963, Ca^{++} uptake by fragments of rat liver mitochondria and its dependence on electron transport, *J. Biol. Chem.*, **238**, 1841.

VASINGTON, F. D., and MURPHY, J. V., 1962, Ca^{++} uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation, *J. Biol. Chem.*, **237**, 2670.

WEINBACH, E. C., and VON BRAND, T., 1965, The isolation and composition of dense granules from Ca^{++}-loaded mitochondria, *Biochem. and Biophysic. Research Commun.*, **19**, 133.

WITTER, R. F., WATSON, M. L., and COTTONE, M. A., 1955, Morphology and ATPase of isolated mitochondria, *J. Biophysic. and Biochem. Cytol.*, **1**, 127.